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(54) Title: CONJUGATES OF ANTISENSE OLIGONUCLEOTIDES AND THERAPEUTIC USES THEREOF (57) Abstract Conjugates consisting of one or more antisense oligonucleotide(s) bound to a ligand binding molecule which recognizes a cell surface molecule that are useful for treating diseases are disclosed. The conjugates are preferably used to treat viral infections, AIDS; the prevention or treatment of sepsis; and the treatment or suppression of cancer caused either by cellular oncogene or viral infections. Further disclosed are methods for making the conjugates.		

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CONJUGATES OF ANTISENSE OLIGONUCLEOTIDES AND THERAPEUTIC USES THEREOF

This invention relates generally to the inhibition and/or regulation of expression and replication of cellular or foreign nucleic acid in cells by means of antisense oligonucleotides conjugated to a ligand binding molecule that recognizes a cell surface molecule. More specifically, this invention relates to the inhibition of viral expression or replication in viral infected cells, and/or endogenous nucleotide expression or replication in cells. Specific examples are compositions useful in AIDS, TNF induced diseases/symptoms, and cancer therapy comprising at least one antisense oligonucleotide (or analogue thereof) conjugated to an antibody against a cell surface antigen, a growth factor, an antibody to a growth factor, an antibody to a cell surface receptor, or an antibody which recognizes a complex of growth factor and receptor. Methods of making the compositions, and methods of using the compositions in therapy are also disclosed.

The present invention presents applications of antisense oligonucleotide conjugates for the treatment of diseases, and concerns antisense oligonucleotides, transferrin receptor antibody, interleukins, AIDS, TNF, and leukemia. Background information as to each of these is presented below.

Antisense Oligonucleotide

The antisense oligonucleotide is a single-stranded nucleic acid, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA-RNA, a DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are complementary to the sense or coding strand of the gene. Recently, formation of a triple helix has proven possible where the oligonucleotide is bound to a DNA duplex. It was found that oligonucleotides could recognize sequences in the major groove of the DNA double helix. A triple helix was formed thereby. This suggests that it is possible to synthesize sequence-specific molecules which specifically bind double-stranded DNA via recognition of major groove hydrogen binding sites. Le Doan, T. et al., 1987, Nucleic Acid Res. 15 749.

By binding to the target nucleic acid, the above oligonucleotides can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking the transcription, processing, poly(A) addition, replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradations.

The antisense oligonucleotide can be used to selectively suppress certain cellular functions. For example, in oncogenic transformed cells, oligonucleotides complementary to the oncogene suppresses its expression. An antisense oligonucleotide has been shown to inhibit c-myc protein expression in a human
5 promyelocytic leukemia cell line, HL60, which over expresses the c-myc protooncogene. The antisense oligonucleotide used was complementary to regions of the c-myc mRNA. Wickstrom E.L., et al., 1988, PNAS (USA), 85:1028-1032 "Human Promyelocytic Leukemia HL60 cell Proliferation and c-myc Protein Expression are Inhibited by an Antisense Pentadecadeoxynucleotide Targeted Against c-
10 myc mRNA". See also Harel-Bellan, A., et al., 1988, Exp. Med., 168:2309-2318 "Specific Inhibition of Lymphokine Biosyntheses and Autocrin Growth Using Antisense Oligonucleotides in Th1 and Th2 Helper T-cell clones".

Antisense oligonucleotide can also be used to inhibit replication and expression of nucleic acid foreign to the host cells. Antisense oligonucleotides are prepared in the
15 laboratory and then introduced into cells, for example, by microinjection, uptake from the cell culture medium into the cells, or expressed in cells after transfection with plasmids or retroviruses carrying an antisense gene. Antisense oligonucleotides were first discovered to inhibit the following viral replication or expression in cell culture: Rous sarcoma virus, vesicular stomatitis virus, herpes simplex virus type 1, simian
20 virus, and influenza virus. Since then, inhibition of mRNA translation by antisense oligonucleotides has been studied extensively in cell-free systems including rabbit reticulocyte lysates and wheat germ extracts. Inhibition of viral function by antisense oligonucleotides has been demonstrated *in vitro* using oligonucleotides which were complementary to the AIDS HIV retrovirus RNA. Goodchild, J., 1988, "Inhibition of
25 Human Immunodeficiency Virus Replication by Antisense Oligodeoxynucleotides", Proc. Natl. Acad. Sci. (USA), 85 (15):5507-11. The Goodchild study showed that oligonucleotides that were most effective were complementary to the poly(A) signal; also effective were those targeted at the 5' end of the RNA: the cap and 5' untranslated region, next to the primer binding site and at the primer binding site. The cap, 5'
30 untranslated region, and poly(A) signal lie within the sequence repeated at the ends of retrovirus RNA (R region) and the oligonucleotides complementary to these might bind twice to the RNA.

Toxicity studies in mice have shown that antisense oligodeoxynucleotides inhibit HIV only at high levels. Goodchild, J., *Id.* This is due in part to limited cell
35 permeation of some derivatives. Because the oligonucleotides are expensive, the high dosage level necessarily increases the cost of treatment. As a result, research into modified oligonucleotides has been conducted. Examples of such modifications

include: terminal blocking groups, internucleoside methylphosphonates or phosphorothioates, α -analogues, covalent addition of polylysine or other ligands, (e.g. attachment of iron EDTA or have analogs to the oligonucleotides), acridine or its derivatives, and other intercalating agents. See e.g. Stein, C. A. et al, 1988, *Cancer Res.*, **48**: 2659-2668, "Oligonucleotides as Inhibitors of Gene Expression: A Review"; Helene, C., 1989, *Br. J. Cancer*, **60**:157-160, "Artificial Control of Gene Expression by Oligodeoxynucleotides Covalently Linked to Intercalating Agents." Also included are carbamate linked nucleic acid analogs, and antisense 2'-OMe RNA oligonucleotide. Stirchak, E.P., et al., 1989, *Nuc. Acids Res.*, **17**(15):6129-6141, entitled "Uncharged Stereoregular Nucleic Acid Analogs: 2. Morpholino Nucleoside Oligomers with Carbamate Internucleoside Linkages"; Lamond, A.I., et al., 1989, *Cell*, **58**:383-390, "Probing the Structure and Function of U2 snRNP with Antisense Oligonucleotides made of 2'-OMe RNA".

Sequence-specific suppression of viral expression in T-cells chronically infected with human immunodeficiency virus 1 (HIV-1) has been demonstrated using antisense phosphorothioate oligodeoxynucleotides. Matsukura, M., 1989, "Regulation of Viral Expression of Human Immunodeficiency Virus in Vitro by an Antisense Phosphorothioate Oligodeoxynucleotide Against Rev (art/trs) in Chronically Infected Cells", *Proc. Natl Acad Sci. (USA)*, **86**:4244-48. Oligodeoxynucleotide methylphosphonates have also been demonstrated to inhibit HIV expression. Sarin, P.S., 1988, "Inhibition of Acquired Immunodeficiency Syndrome Virus by Oligodeoxynucleotide Methylphosphonates", *Proc. Natl. Acad. Sci. (USA)*, **85**(20):7448-51.

Recently, there has been research into the formation of a triple helix consisting of oligonucleotide bound to a DNA double helix. See Helene, *supra*. Triple helix formation opens new possibilities to control gene expression at the transcriptional level. For example, it has been reported recently that myc gene transcription could be inhibited *in vitro* by a purine-rich DNA oligonucleotide recognizing a sequence upstream of the transcription initiation site, and this is thought to result from triple helix formation. Cooney, M. C. et al., 1988, *Science*, **241**:456-459. Cooney further postulated that it would be possible for a RNA to bind to a duplex DNA.

Anti-transferrin Receptor Antibody and Interleukin

Transferrin is the major iron transport protein found in human blood. When transferrin binds to the transferrin receptor found on the cell surface, the transferrin is internalized. When transferrin receptor antibodies bind to the transferrin receptor, they

are also internalized into the cell through a highly efficient coated pit pathway. Fitzgerald, D.J.P., et al., 1983, "Enhancement of Toxicity of Anti-Transferrin Receptor Antibody-Pseudomonas Exotoxin Conjugates by Adenovirus", Proc. Natl. Acad. Sci. (USA), 80:4134-4138.

5 Interleukin is produced by T-cells, and other cell types. For example, interleukin 2 (IL 2, T-cell growth factor) is produced by T-lymphocytes after antigen- or mitogen-stimulation and is required for the proliferation of activated T-cells. IL 2 is an essential mediator of the immune response. Paetkau, V., 1981, Nature, 294:689, "Lymphokines on the Move"; Ruscetti, F. W. et al., 1981, Blood, 47:379, "Human T-
10 Lymphocyte Growth Factor: Regulation of Growth and Function of T-Lymphocytes". There is also preliminary evidence that it may be responsible for the abnormal cell proliferation in human lymphoblastic leukemias. Gillis, S. et al., 1980, AACR, Abstract No. 955:238, "Correlation of Elevated Terminal Transferase Activity (TdT) with Production of T-Cell Growth Factor (TCGF) in Human Leukemia Cells"; Venuta
15 S. et al., "Production and Regulation of Interleukin-2 in Human Lymphoblastic Leukemias Studied with T-Cell Monoclonal Antibodies", (submitted). Activated T-cells, for example, T-cells activated by lipopolysaccharide, have IL-2 receptors.

AIDS

Human T-cell leukemia-lymphotropic virus (HTLV) refers to a family of T-cell
20 tropic retroviruses. Such viruses, which have a role in causing certain T-cell neoplasms, are presently divided into three main types or subgroups: (1) HTLV-type I (HTLV-I), which appears to cause adult T-cell leukemia-lymphoma (ATLL); (2) HTLV-type II (HTLV-II), which has been isolated from an individual having a T-cell variant of hairy cell leukemia; and (3) HTLV-type III (HTLV-III), which has been identified as
25 the etiologic agent of acquired immune deficiency syndrome (AIDS). HTLV-III is also known as lymphadenopathy-associated virus (LAV), AIDS related virus (ARV) and human immunodeficiency virus (HIV). (The HTLV-III virus will hereinafter be referred to as HIV or AIDS virus.) Popovic, M., et al., 1984, Science, 224:497-500; Gallo, R.C., et al., 1984, Science, 224:500-503; Wong-Staal, F. and Gallo, R.C.,
30 1985, Nature, 317:395-403; and Curran, J.W., et al., 1985, Science, 229:1352-1357.

AIDS was first recognized in 1981 and since that time, the disease has come to be recognized as a new epidemic. RNA Tumor Viruses (2d edition), 2:437-443, Cold Spring Laboratory, 1985.

HIV infection is associated with the development of the clinical syndrome of
35 AIDS. The following may be an explanation of the mechanism by which HIV operates. HIV appears to attack helper T-cells (T-lymphocytes or OKT4-bearing T-

cells as they are sometimes known, and macrophage), monocytes, and possibly other human cells, e.g., certain cells within the brain. The invaded helper T-cells when activated become HIV producers. The helper T-cells are quickly destroyed and their number is depleted to such an extent that the body's B-cells and other T-cells normally stimulated by helper T-cells no longer function normally or produce sufficient lymphokines and antibodies to destroy the invading virus or other invading microbes, etc.

While the HIV virus does not necessarily cause death directly, it does in many cases cause the immune system to be so severely depressed that the infected individual is infected or afflicted with various other diseases, that are often life threatening, such as herpes, cytomegalovirus, Kaposi's sarcoma and Epstein-Barr virus related lymphomas. Thus, much effort is being put into developing methods of detecting the presence of HIV in body tissues and fluids (e.g., blood, saliva), developing vaccines, and prophylactics and therapeutics. So far, only one drug AZT (3' -Azido- 3' - deoxythymidine) has been approved by the United States Food and Drug Administration to treat AIDS. AZT has serious side effects and limited efficiency. Therefore, there remains a need for a method for preventing the disease or for treating those who become infected with the virus. However, current efforts to develop a broad spectrum anti-HIV vaccine may be seriously compromised, in light of the variation in envelope proteins (which are the principal antigenic determinants of the virus) observed among various strains of HIV. Hahn, G.H., *et al.*, 1985, PNAS (USA), 82:4813-4817; Benn, S., *et al.*, 1985, Sciences, 230:949-951. Other methods of blocking the effects of the virus are clearly needed.

TNF Induced Diseases/Symptoms

Endotoxin, a lipopolysaccharide (LPS) component of the cell wall of certain bacteria often causes the infected mammal to develop sepsis characterized by hypotension, disseminated intravascular coagulation, renal, hepatic, and cerebral injury, which eventually lead to the death of the mammal. The mechanism of action of LPS is unclear. It is widely believed that after its introduction, LPS stimulates macrophage cells to produce tumor necrosis factor (TNF) and other factors. Recent work implicates TNF as a cause of many negative effects associated with sepsis. Injection of TNF in mice and other animals mimics the effects of LPS and causes tissue injury, shock and death. However, other studies imply that at low doses, TNF is beneficial to the infected animal to combat infection. For example, it had been found that mice injected with TNF were more resistant to certain forms of malaria. Further, TNF had been shown to stimulate macrophages and other blood cells to kill the

parasites that cause Chaga's disease and schistosomiasis. However, in another study, TNF appeared to mediate a lethal inflammation of the brain in mice infected with malaria. The mice could be protected by antibody that neutralizes the brain damaging effect of TNF. Further, human patients with severe meningococcal infections and relatively high levels of the TNF in their blood were more likely to die from sepsis than patients with no detectable levels of the polypeptide. Old, L.G., 1988, Scientific America, May:59-75, "Tumor Necrosis Factor".

Additionally, TNF has been shown to be involved in initiating the expression of human immunodeficiency virus in human cells that carry latent virus. Folks, et al., 1989, PNAS (USA), 86:2365. Therefore, it appears that the prevention of or inhibition of TNF production would serve as a valuable prophylactic for the treatment of AIDS patients by preventing the expression of virus that is latent in the patient.

In summary, it appears that TNF is helpful in the local control of injury and infection, but may also be toxic when released in large amounts or at particularly sensitive sites. Therefore, research has been conducted to block TNF's action where its effects becomes more detrimental than protective. To date, the research has centered around anti-TNF antibody Tracey, et al., 1987, Nature, 330:662, and molecules with TNF inhibitory activity such as those present in the urine of febrile patients. Seckinger, et al., 1988, J. Exp. Med., 167:1511. However, the particular non-antibody TNF inhibitor has not been purified and characterized to the point where it is clinical useful. The anti-TNF antibody involved non-human TNF and non-human TNF antibody tested so far posed the problem of immunologic rejection in humans.

Myeloid and Acute Lymphocytic Leukemias

A significant number of patients with chronic myeloid leukemia (CML) and acute lymphocytic leukemia (ALL) exhibit a cytogenetic abnormality known as Philadelphia (Ph⁺) chromosome. Kawasaki, E.S., "Diagnosis of Chronic Myeloid and Acute Lymphocytic Leukemias by Detection of Leukemia-Specific mRNA Sequence Amplified In Vitro", 1988, PNAS (USA), 85:5698-5702. The Philadelphia translocation fuses the BCR and ABL genes, resulting in the expression of leukemia-specific chimeric BCR-ABL messenger RNAs. Oligodeoxynucleotides complementary to this unique fusion sequence have been shown to hybridize to the BCR-ABL mRNA. The specific sequences of the oligonucleotides are as follows:

In the case of CML, the oligonucleotide, GCTGAAGGGCTT⁺TTGAACTCTGCTTA hybridizes to BCR exon 3/ABL and exon II junction sequences; and oligonucleotide GCTGAAGGGCTT⁺CTTCCTTATTGATG hybridizes to BCR exon 2-ABL and exon II fusions. In the case of ALL,

oligonucleotide GCTGAAGGGCTT[^]CTGCGTCTCCAT hybridizes to the junction of BCR/ABL. In the above sequences, the arrows denote the junction between BCR and ABL exons.

5 In summary, there is a need to identify and develop methods and compositions for treating diseases that have hitherto been intractable, particularly AIDS, sepsis, and certain forms of cancer. The instant invention describes a method applicable for the treatment of these diseases.

10 This invention relates generally to the inhibition and regulation of expression of foreign and cellular RNA or DNA in cells by means of antisense oligonucleotides conjugated to a ligand binding molecule that recognizes cell surface molecules. The antisense oligonucleotide used in the conjugate of this patent application includes oligonucleotides, which bind to single stranded nucleic acid and those which bind a strand of a double stranded nucleic acid. The double stranded nucleic acids include DNA/DNA, a DNA/RNA, and RNA/RNA. These antisense oligonucleotides include
15 unmodified and modified nucleic acids, discussed *supra*, in the "Background of the Invention" section. Further, as used in this patent application, the oligonucleotide can be either an oligoribonucleotide or a oligodeoxyribonucleotide.

More specifically, this invention relates to the inhibition of viral expression or replication in viral infected cells, and endogenous nucleic acid function in cells.
20 Specific examples are compositions useful in AIDS, TNF induced diseases/symptoms and cancer therapy comprising antisense oligonucleotides conjugated to antibody against cell surface antigen, growth factors, antibody to growth factors, antibody to growth factor cell surface receptors, and antibody to a complex comprising growth factor and growth factor receptor. Also shown are methods of making the
25 compositions, and methods of using the compositions in therapy.

Figure 1 presents the sequence of the oligonucleotides BB01, BB02, and BB03.

Figure 2 presents the sequence of the oligonucleotides BB04, BB05, and BB06.

30 Figure 3 graphically presents competitive binding data comparing the 454A12-oligonucleotide conjugates with 454A12-rRA immunotoxin.

Figure 4 presents the result of the first experiment testing the efficacy of the 454A12-oligonucleotides for inhibiting HIV viral functions.

35 Figure 5 presents the result of the second experiment testing the efficacy of the 454A12-oligonucleotides for inhibiting HIV viral functions.

Figure 6 presents the location of the TNF sense sequences on HuTNF cDNA which can serve as the templates for antisense oligonucleotides in a conjugate with

antibodies for inhibition or suppression of TNF induced diseases/symptoms, for example, sepsis.

The invention described herein presents conjugates comprising a ligand binding molecule and one or more antisense oligonucleotides bound to the ligand binding molecule. These conjugates present a more efficient means than found in the prior art, for the delivery of antisense oligonucleotides into cells, that is via attachment to a cell surface targeting molecule. Due to low cell permeation, the prior art shows that high levels of antisense oligonucleotides are required to be effective. Because oligonucleotides are expensive, the invention enables a more effective and less costly use for the oligonucleotides. Additionally, because the oligonucleotides are cell targeted, they spend less time exposed to extra-cellular nucleases, which may degrade the oligonucleotides. For degradatory effect of extracellular nuclease on oligonucleotides, see Tidd, D.M., et al., 1989, Br. J. Cancer, 60:343-350, "Partial Protection of Oncogene, Anti-sense Oligonucleotides Against Serum Nuclease Degradation Using Terminal Methylphosphonate Groups".

Further, unlike other conjugates, for example immunotoxins, which are toxic if they permeate non-targeted cells, conjugates consisting of antisense oligonucleotide and ligand binding molecule have the advantage of being non-toxic to non-targeted cells. This is because the antisense oligonucleotide specifically suppresses or inhibits only the targeted complementary nucleic acid, leaving non-targeted nucleic acid and therefore the non-targeted cells unharmed. The antisense oligonucleotide used in the conjugate of this patent application includes oligonucleotides, which bind to single stranded nucleic acid and those which bind to double stranded nucleic acid. The double stranded nucleic acids include DNA/DNA, a DNA/RNA, and RNA/RNA. These antisense oligonucleotides include unmodified and modified nucleic acids, discussed supra, in the "Background of the Invention" section. Further, as used in this patent application, the oligonucleotide can be either an oligoribonucleotide or a oligodeoxyribonucleotide. The conjugate may be produced from its individual components as described below.

The invention described below draws on previously published work in molecular biology/biochemistry. By way of example, such work consists of scientific papers, patents or pending patent applications. All of these publications, those described previously as well as those described below are hereby incorporated by reference.

Synthesis of the Oligonucleotides and Addition of Sulfhydryl Group Thereto

Oligonucleotides may be synthesized according to standard methods known in the art. For example, one method for the synthesis and characterization of the

oligodeoxynucleotides is discussed in Goodchild, J., *supra*. The oligonucleotides with reactive sulfhydryl groups can also be synthesized on an automated DNA synthesizer according to methods known in the art. Several references teach methods of introducing a sulfhydryl group at the 5' terminus of synthetic oligonucleotides. For example, Connolly, 1985, Nuc. Acids Res. 13(12):4485-4502, describes a method of incorporating a sulfhydryl moiety into synthetic DNA using S-trityl-O-methoxymorpholinophosphate derivatives of 2-mercaptoethanol, 3-mercaptopropan-1-ol and 6-mercaptohexan-1-ol. Connolly further describes derivatization of the sulfhydryl-containing oligonucleotide with thiol-specific probes. Additionally, a new and improved procedure can be used and is described in detail in PCT patent application, PCT/US85/03212, Levenson, C., *et al.*, "Oligonucleotide Functionalizing Reagents and Methods", filed September 19, 1988.

Ligand Binding Molecules

The ligand binding molecule includes any cell surface recognizing molecule. It can include any molecule with a specific affinity for a cell surface component. The cell surface component can be those generally found on any cell type. Preferably, the cell surface component is specific to the cell type targeted. More preferably, the cell surface component also provides a pathway for entry into the cell, for the oligonucleotide-ligand binding molecule conjugate or the oligonucleotide attached thereto. Preferably, the conjugation of the oligonucleotide to the ligand molecule does not substantially interfere with the ability of the ligand binding molecule for binding to the cell surface molecule or for entry of the conjugate or oligonucleotide into the cell. More preferably, the ligand binding molecule is a growth factor, an antibody to a growth factor, or an antibody to a cell surface receptor. Alternatively, the ligand binding molecule is an antibody which recognizes a complex of growth factor and receptor.

In the preferred embodiment, the ligand binding molecule is an antibody or antigen binding fragment derived therefrom. Antibody, either polyclonal, monoclonal, bispecific, etc., may be generated by methods well known in the art. The more preferred embodiment antibody is transferrin receptor antibody. The preferred transferrin receptor antibody is described in EPA 226,419, "Anti-human Ovarian Cancer Immunotoxins and Methods of Use Thereof", published June 24, 1987, applicants Bjorn, M. J. *et al.*, and denoted 454A12, therein. (Samples of the hybridomas which produce the monoclonal antibody had been deposited with the Collections of In Vitro International under the accession number IVI 10075. This deposit was made under the Budapest Treaty and will be maintained and made accessible according to the provisions thereof.) The 454A12 binds to transferrin

receptors, but does not substantially inhibit binding of transferrin to the transferrin receptor. It is thus different from the transferrin monoclonal antibodies claimed in U.S. Patent No. 4,434,156, which inhibit binding of transferrin to the transferrin receptor. Both types of transferrin antibody are suitable ligand binding molecules.

5 Method for Conjugating the Oligonucleotides and Ligand Binding Molecule

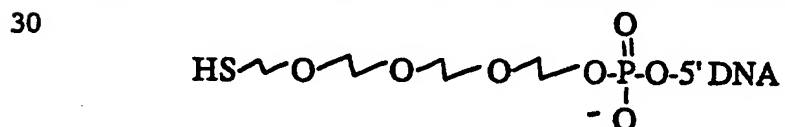
Oligonucleotides can be conjugated to the ligand binding molecule for example, through disulfide, amides, or thioethers bonds, or through peptide linker, or any other type of chemical bond that is sensitive to cleavage in lysosomes by either enzymes or the acidic environment.

10 The preferred embodiment conjugate comprises ligand binding molecule and oligonucleotide bound by a disulfide bond. Exemplary of this approach is binding the preferred antibody 454A12 to oligonucleotides by a disulfide bond.

Oligodeoxynucleotides with the following sequences were used:

CTGGTCTAACCAGAGAGACC (designated BB01); and
 15 GCAAGCTTTATTGAGGCTTA (designated BB02). The control has the following sequence: CAGTCAGTCAGTCAGTCAGT (designated BB03). BB01 was complementary to the cap or initiator codon and BB02 was complementary to the poly(A) signal of the HIV genomic RNA. The cap and poly(A) signal lie within the sequence repeated at the ends of the HIV RNA (R region). BB03 is a 20-mer not
 20 complementary to the HIV RNA. BB03 has been tested and shown to be inactive. Goodchild, J., *supra*. All three oligonucleotides did not have a reactive sulfhydryl group. They, therefore, could not form a disulfide conjugate with the transferrin receptor antibodies. Figure 1 presents the sequences of the oligonucleotides.

In order to form a conjugate, oligonucleotides with a reactive sulfhydryl group
 25 were synthesized. In the preferred embodiment, the oligonucleotides with reactive sulfhydryl groups were designated BB04, BB05, and BB06, each with a sequence corresponding to BB01, BB02, and BB03 respectively. Figure 2 presents the sequences and data on these oligonucleotides. The oligonucleotides had the following generalized structure (the sulfhydryl group was denoted as X in Figure 2):



At the same time, a sulfhydryl group was added to 454A12. The oligonucleotide was covalently linked to the 454A12 through a disulfide exchange reaction between the
 35 sulfhydryl groups of the two compounds. This reaction is described below.

Addition of Sulfhydryl Group on the Antibody

The addition of a thiol group or an activated disulfide group to an immunoglobulin is known in relation to the synthesis of immunotoxins. U.S. Patent No. 4,340,535, Jul. 20, 1982, Voisin *et al.*, "Cytotoxic Products Formed by Covalent Bonding of the A Chain of Ricin with an Antibody and the Process for Their Preparation and Use." The procedures disclosed therein regarding addition of the thiol group to the antibody is incorporated hereby.

In our preferred example, Ellman's reagent was added to a solution of 454A12 antibody in 40 mM phosphate buffer containing 1 mM EDTA (final concentration of 454A12 was 3.2 mg/ml, and that of Ellman's reagent was 1mM. Final pH of the mixture was 8). The reaction was allowed to proceed for 30 minute at room temperature. At the end of 30 minutes, the reaction mixture was cooled in an ice bucket to 4°C and a ten fold excess of 2-iminothiolane reagent was added. The reaction mixture was allowed to continue at 4°C overnight. At the end of the reaction, the excess reagents were separated on a 1.5 x 15 cm column of Sephadex G-25 equilibrated with 40 mM sodium phosphate, pH 7.6, containing 0.2 M NaCl and 1mM EDTA.

Disulfide Linkage of the Oligonucleotide with the Antibody

The derivatized antibody was then covalently linked, in a disulfide exchange, to the sulfhydryl group on the oligonucleotide. This linkage was achieved by incubating the two components (454A12-IT-TNB at 4 nM and oligonucleotide at 100 nM, final concentration) overnight at 4°C. The sample turned yellow, indicating that the TNB group was being released and the desired product was being formed.

The sample was then passed through a 2.5 x 24 cm column of Sephadex G-25 resin equilibrated with 40 mM sodium phosphate, pH 7.6, containing 0.2 M NaCl. The separation profile of a standard mixture of proteins was compared to 454A12 which elutes at 20.3 minutes. The reaction mixture containing the conjugate 454A12-BB06, was run on GF250 preparation HPLC in PBS, pH 7.6. The peak eluting at 17.7 minutes was shown by SDS-PAGE analysis to contain protein with molecular sizes larger than the unconjugated antibody. Reaction mixtures containing 454A12-BB04 and 454A12-BB05 were also shown by SDS-PAGE analysis to contain protein with molecular sizes larger than the unconjugated antibody.

Analysis of the Conjugates for Nucleic Acid Content

To determine the nucleic acid content of the conjugates, their absorbance at 260 and 280 nm were observed. For this purpose, the conjugates, each in 40 mM sodium phosphate, pH 7.6, containing 0.2 M NaCl presterilized by filtration was used.

5 The result was as follows:

	<u>Conjugate</u>	<u>Absorbance</u>		
		260	280	280/260
	BB04	0.665	0.477	0.72
	BB05	0.719	0.497	0.69
10	BB06	1.420	0.980	0.69

Based on the above data, it was calculated that about 4 to 8 oligonucleotides were conjugated to one antibody. It is possible that higher number of oligonucleotides can be conjugated to each antibody. It should be noted that oligonucleotides of different sequences can be conjugated to a single ligand binding molecule. This is especially desirable for treatment of AIDS where different strains of the HIV virus exist in a single patient.

Binding Efficiency of the Conjugate to Transferrin Receptors

It is important that conjugation of the oligonucleotide to the ligand binding molecule not destroy the binding properties of the latter. To demonstrate that the conjugation did not destroy the ability of 454A12 to bind to the transferrin receptor on cell surfaces, the oligonucleotide conjugates were tested for the ability to protect cells against the effects of an immunotoxin consisting of 454A12 and recombinant ricin A chain. The result showed that the immunotoxin alone inhibited protein synthesis by 50% at a concentration 0.01 nM. When the same experiment was done in the presence of 30 nM BB04 and BB05 conjugates, 10 fold higher concentrations of immunotoxin were required to reach 50% inhibition (See Figure 4). The results showed that the conjugates compete with the immunotoxin for the transferrin binding sites. Therefore, conjugation of 454A12 to the oligonucleotides did not cause detectable damage to the antibody binding site. The immunotoxin 454A12-rRA was made as described in EP 226,419, published June 24, 1987, to Bjorn, M.J. *et al.*, entitled "Anti-human Ovarian Cancer Immunotoxins and Methods of Use Thereof".

Efficacy of the Conjugates to Inhibit HIV Viral Expression

To test the efficacy of the above described conjugates in inhibiting HIV viral expression, the following procedures/experiments were carried out.

1. Isolation and Culture of Peripheral Blood Monocytes (PBMC) and Virus Isolation by Monocyte Cocultivation

To test the effect of the conjugates it was desirable to have a source of HIV infected monocytes. Monocytes infected with HIV virus were produced in the following manner. Monocytes treated with rCSF-1 and maintained in culture for at least 7 days were used for cocultivation experiments with freshly isolated PBMC from seropositive HIV-infected individuals. The detailed procedures for isolation and culture of peripheral blood monocytes and virus isolation by monocyte cocultivation are described in Gendelman, H.E., et al., 1988, "Efficient Isolation and Propagation of Human Immunodeficiency Virus on Recombinant Colony-Stimulating Factor 1-Treated Monocytes", J. Exp. Med., 167:1428-1441.

15 Effect of Conjugates on HIV

Reverse transcriptase activity was assayed as an indicator of viral replication. Inhibition of viral replication was reflected in reduced reverse transcriptase activity. Assays of virion-associated reverse transcriptase (RT) activity were performed with [³²P] deoxythymidinetriphosphate in a protocol described in detail in Willey, R. L., 1988, J. Virol., 62: 139-147, "In Vitro Mutagenesis Identifies a Region within the Envelope Gene of the Human Immunodeficiency Virus that is Critical for Infectivity." The efficacy of the 454A12-oligonucleotides for inhibiting HIV viral functions was determined. (Figures 5 and 6 present the results, respectively). The experiments used unconjugated oligonucleotides (without sulfhydryl group), mixtures of unconjugated oligonucleotides (without sulfhydryl group) plus 454A12, and conjugates comprising 454A12 and oligonucleotides bound by a disulfide bond. In the mixtures of unconjugated oligonucleotides with antibodies, the oligonucleotides were prevented from conjugating with the antibodies because the oligonucleotides lacked the sulfhydryl group necessary for linkage with the antibodies.

30 In the experiments, each sample was introduced at the same time the monocytes were infected with the HIV virus, and every three days thereafter along with a fresh change of media. The monocytes were infected with HIV at 100 viral infective units (1 unit being the amount which results in 50% cell infection after 1 month in culture). The reverse transcriptase levels (in count per minute per 0.01 ml) in the culture fluid were

measured after twelve days of cocultivation. In the first experiment, the following samples were used:

Sample	Material
1	oligonucleotide without sulfhydryl group, BB01
5 2	oligonucleotide without sulfhydryl group, BB02
3	oligonucleotide without sulfhydryl group, BB03 (Control)
4	mixtures of unconjugated BB01 and antibody 454A12
5	mixtures of unconjugated BB02 and antibody 454A12
6	mixtures of unconjugated BB03 and antibody 454A12
10 7	conjugate comprising BB04 and antibody 454A12
8	conjugate comprising BB05 and antibody 454A12
9	conjugate comprising BB06 and antibody 454A12
10	buffer (as used in samples 1 to 9)
Medium	culture infected with HIV
15 Background	culture alone, without HIV, oligonucleotide nor antibody

Samples 1 to 6 contained 50 µg/ml of oligonucleotides at 1X concentration. (Based on a reading of 1.6 at OD₂₆₀; a reading of 32 at OD₂₆₀ being indicative of 1 mg/ml of oligonucleotide.) Samples 7 to 9 contained 19 µg/ml of oligonucleotide at 1X concentration (based on a reading of 0.6 at OD₂₆₀). As indicated in Figure 5, 1/100, 1/1000, and 1/10,000 concentrations of the samples were used, respectively.

The results showed that the unconjugated oligonucleotide BB01 of Sample 1 had some inhibitory activity, but the oligonucleotide-antibody conjugates of Samples 7 and 8 clearly inhibited the production of reverse transcriptase.

The second experiment followed the same protocol as the first and tested some of the samples at 1/10,000 concentration. The results of the second experiment indicated the oligonucleotide-antibody conjugate (Sample 7) was more effective in inhibiting viral expression than its counterpart, the unconjugated oligonucleotide (Sample 1). Further, the oligonucleotide-antibody conjugate (Sample 8) was more effective at inhibiting viral expression than the unconjugated oligonucleotide mixed with free antibody (Sample 5). This result showed that the increase in inhibition of viral expression was due to conjugation of the oligonucleotide with the antibody. The control, non-complementary oligonucleotide mixed with free antibody (Sample 6), showed a higher reading of reverse transcriptase than the conjugated non-control oligonucleotide antibody conjugates (Samples 7 & 8) (except for days 5, where the reading for Samples 7 and 6 were the same). These experiments establish that oligonucleotide antibody conjugates can be used to suppress HIV expression.

Alternative Conjugation Method

Alternatively, the oligonucleotide can be conjugated to the antibody using heterobifunctional crosslinkers. The preferred crosslinkers are maleimide active esters, and encompass a family of crosslinkers that produce a thioether bond linking antibody and oligonucleotide consisting of an activated carboxylate, a spacer molecule that contains ether groups, and a maleimido group to which a thiol on the oligonucleotide can bind. The linker will have a preferred length of up to about 34 angstroms; however, longer linkers are intended to come within the scope of the invention. These linkers, referenced to as malsac HNSA glut, are described in U.S. patent application, Serial No. 217,938, filed July 12, 1988, to L. Houston, et al., entitled "Thioether Linked Immunotoxin Conjugates". The procedure is described herein. Using malsac HNSA glut, the conjugation consists of reacting antibody having a free amino group with a maleimide-active ester crosslinker in a suitably buffered solution. Next, the derivatized antibody is separated from the crosslinker. The isolated derivatized antibody can then be reacted with the oligonucleotide having a sulfhydryl group.

In more detail, the thioether bond that links the antibody and the oligonucleotide described herein result from heterobifunctional crosslinkers having two reactive groups, an active ester designed to react primarily with amino groups, preferably on the antibody molecule; and a maleimido group that reacts with sulfhydryl groups, preferably present on the oligonucleotide. It is important to note, however, that by suitable chemical modification of antibody or oligonucleotide that reaction of antibody can be made to occur with the maleimido group, and reaction of oligonucleotide with the active ester. For example, antibody or antibody fragments can be prepared having free sulfhydryl groups by techniques well known in the art. Particularly useful is the procedure shown in Urnovitz, U.S. Patent 4,698,420, which is hereby incorporated by reference. Certain classes of antibody, specifically IgM and IgA, exist as aggregates such that antibody molecules are joined together by disulfide bonds. Reduction of the aggregates causes the formation of individual antibody molecules having free sulfhydryl groups which can be reacted with the maleimido group of the instant heterobifunctional crosslinkers. Similarly, antibody fragments can be produced using suitable enzymes, and reduced thereby rendering sulfhydryl groups available for reaction. Alternatively, in lieu of reducing antibody or antibody fragments to obtain a reactive sulfhydryl group, sulfhydryl group(s) can be introduced into these molecules by reactions described in U.S. Patent Nos. 4,350,626, 4,450,154, and 4,340,535, which are hereby incorporated by reference. Regardless of how the sulfhydryl group is realized, antibody, or antibody fragment, is reacted with the heterobifunctional crosslinker at pH's which favor sulfhydryl maleimide reaction, preferably about pH 6.

Synthesis of Heterobifunctional Crosslinkers

The heterobifunctional crosslinkers of the present invention consist of an activated carboxylate, a spacer containing ether groups, and a maleimido group. The initial step in the synthesis of this type of crosslinker consists of employing an ether containing spacer group, preferably an ether diamine, having amino reactive groups on both ends of the spacer, with a reversible amino protective reactive group to block one of the amino groups. Next, the remaining amino group is reacted with a dicarboxylic anhydride; glutaric anhydride and succinic anhydride can be employed, among others. The protecting group is then removed, preferably by exposure to acid, and the deprotected amino group reacted with an active ester of maleimido to introduce a maleimide functionality at this region of the spacer. At this point in the synthesis the spacer contains a maleimido group at one end, and a group capable of forming an active ester, that is to say, a carboxylic acid, at the other. This molecule is then reacted with 1-hydroxy-2-nitrobenzene-4-sulfonic acid in the presence of a suitable condensing agent to yield the maleimide active ester. It will, of course, be understood by those skilled in the art that each of the reactions described above is followed by suitable purification procedures.

It is important to note that spacers of various length can be employed to produce the instant crosslinkers. The preferred spacer has a length of about 34Å; however, additional spacers considerably shorter or longer in length can be used and are available from Texaco Chemical Company under the trade name Jeffamine.

The synthesis of the heterobifunctional crosslinkers involves initially reacting the diamino containing spacer with the protecting group, 2-tert-butoxycarbonyloxymino-2-phenyl-acetonitrile, (BOC). Suitable chromatographic techniques permit the isolation of the mono-BOC-spacer-NH₂ molecule. The preferred dicarboxylic anhydride, glutaric anhydride, is reacted with the mono-BOC-spacer. Suitable chromatographic isolation of the reactants yields BOC-spacer-glutarate. The BOC group is removed, generally using trifluoroacetic acid, the acid removed and the product reacted with a suitable active maleimido ester, preferably maleimido-6-aminocaproyl-ester of 1-hydroxy-2-nitrobenzene sulfonic acid, to yield maleimido-6-aminocaproyl-spacer-glutarate, that hereafter is abbreviated mal-sac-spacer-glutarate. Finally, the heterobifunctional crosslinker is produced by reacting 1-hydroxyl-2-nitrobenzene-4-sulfonic acid with mal-sac-spacer-glut in the presence of a suitable condensing agent, such as dicyclohexylcarbodiimide. Thus, the preferred heterobifunctional maleimide-active ester crosslinker is maleimido-6-aminocaproyl-spacer-glutarate ester of 1-hydroxyl-2-nitrobenzene sulfonic acid, abbreviated as mal-sac-spacer-glut-HNSA.

Synthesis of Maleimido-6-Aminocaproyl-NH-(CH₂)₃-O-(CH₂)₄-O-(CH₂)₃-NH-Glutaryl Ester of 1-Hydroxyl-2-Nitrobenzene Sulfonic Acid

In more detail, the synthesis of the preferred embodiment heterobifunctional crosslinker was conducted as follows.

5 A maleimido active ester crosslinker about 34 Å in length, as measured from the two reactive functional groups of the spacer, was synthesized as follows. Approximately 61.4 grams of the ether diamine, 4,9-dioxa-1,12-dodecanediamine, was dissolved in 600 ml of anhydrous methanol. To this solution was added a slurry containing 81.2 grams of 2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON) in 150 ml methanol. The latter is an amino group protective agent. The mixture was allowed to react overnight, and then concentrated by rotary evaporation, followed by purification on a silica gel column. The mixture was added to the column, and the column first exposed to chloroform : methanol : acetic acid, 80 : 20 : 10. This solvent removed the bis-BOC, or, 4,9-dioxa-1,12-dodecanediamine derivative, and the mono-BOC dodecanediamine derivative was eluted with chloroform : methanol : acetic acid, 10 70 : 30 : 5. Approximately 53.1 grams of a thick oil was produced, and thin layer chromatography using chloroform : acetic acid, 90 : 10 revealed the starting material, 4,9-dioxa-1,12-dodecanediamine to have a R_f value of about 0.08, which was visualized with iodine vapor or ninhydrin. The mono-BOC protected 4,9-dioxa-1,12 20 dodecanediamine had a R_f of about 0.72 and also was reactive with iodine vapor or ninhydrin. High voltage paper electrophoresis having a pH 1.85, and run at 500 volts for 20 minutes revealed that the diamine starting material had a migration distance of 8.8 centimeters and the mono-BOC derivative had a migration distance of about 4.7 centimeters. At a pH 3.5, the diamine starting material migrated 14.4 centimeters and 25 the mono-BOC protected diamine had a migration distance of about 6.1 centimeter.

 The mono protected spacer (BOC-spacer-NH₂) was derivatized to contain an acid reactive group by adding 4.1 grams of BOC-spacer-NH₂ in 40 ml of pyridine, followed by the addition of a two fold molar excess, or 3.08 grams of glutaric anhydride. The reaction was allowed to proceed overnight, followed by removal of the pyridine in vacuo. The residue was taken up in chloroform and extracted three times 30 with 0.5 M aqueous citric acid, followed by three further extractions with saturated, aqueous sodium chloride. The chloroform phase was dried over anhydrous magnesium sulfate and concentrated to a thick oil by rotary evaporation. This resulted in crude BOC-spacer-glutarate (BOC-spacer-glut), which was purified on a silicate gel 35 column in a solvent system comprising chloroform: acetic acid, 90 : 10. About 1.48 grams of a thick oil was recovered, and thin layer chromatography using a developing

solvent of methyl-t-butyl ether: chloroform: acetic acid, 6 : 3 : 1, revealed an iodine and ninhydrin positive species with an R_f of about 0.14, and a second species with an R_f of about 0.49, which also reacted with iodine, but did not react with ninhydrin. The iodine/ninhydrin reactivity profile indicated that the species with the R_f value of 0.14 is BOC-spacer-NH₂, which would be expected to react with both reagents. Ninhydrin reacts with the free amino group. In contrast, the absence of ninhydrin staining with the species having the R_f value of 0.49 is consistent with the formation of BOC-spacer-glut. This was supported by the observation that when the protecting group, BOC, was removed with 6 NHCl, the resulting deprotected molecule was ninhydrin positive.

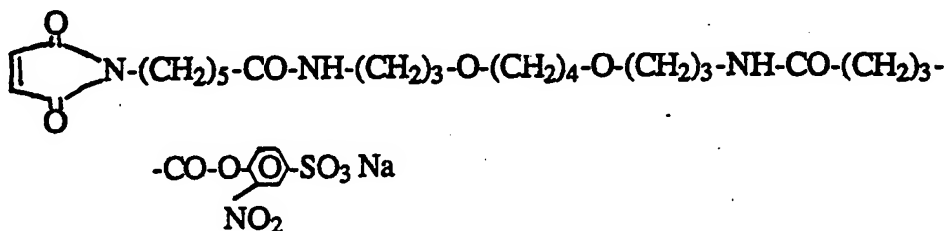
Further characterization of the reaction product revealed that in n-butanol:acetic acid:water, 120 : 30 : 50, BOC-spacer-NH₂ had an R_f of about 0.73 and the BOC-spacer-glut an R_f of about 0.83.

BOC-spacer-glut was treated with trifluoroacetic acid to remove the BOC group. This was carried out by dissolving 1.6 grams of BOC-spacer-glut-OH in 20 ml of 95% trifluoroacetic acid followed by stirring for 30 minutes at room temperature. Trifluoroacetic acid was removed by rotary evaporation, and the residual oil dried overnight *in vacuo*. The resulting deprotected, NH₂-spacer-glut was dissolved in 2.0 ml of dimethylformamide. This solution was neutralized with diisopropylethylamine, followed by adding 2.68 grams of maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitrobenzene-4-sulfonic acid (sodium salt). The reaction was effected at room temperature with continuous stirring. The progress of the reaction was monitored by measuring the formation of the amount of 1-hydroxy-2-nitrobenzene sulfonic acid dianion that results from the aminolysis of the active ester. This is readily achieved by diluting 1 microliter aliquots of the reaction mixture into 5 ml of 0.01 M phosphate buffer, pH 7.0 and reading the absorption of the solution at 406 nm in a spectrophotometer. A method for performing this procedure is described in Aldwin & Nitecki *Analytical Biochemistry*, 164:494 (1987).

After the reaction had gone to completion, generally within about thirty minutes, the mixture was chromatographically purified on a LH-20 Sephadex column (4.5 cm x 40 cm pre-equilibrated in dimethylformamide). Maleimido-6-aminocaproyl-spacer-glutarate (mal-sac-spacer-glut) eluted first from the column. Fractions containing this derivative were pooled, concentrated *in vacuo* and purified using preparative thin layer Chromatotron chromatography. Four mm thick silicate gel plates were employed with the solvent chloroform : methanol : acetic acid, 90 : 10 : 10. A further purification was achieved using high pressure liquid chromatography with a Waters DeltaPrep 3000 HPLC on a microBondapak C18 column and a gradient of acetonitrile in 0.1% aqueous

trifluoroacetic acid. About 0.3 grams of product was obtained. Thin layer chromatography using chloroform, acetic acid, methanol, 90 : 10 : 10 revealed that mal-sac-spacer-glut had an R_f of about 0.66, whereas in the solvent system chloroform : methanol : acetic acid, 70 : 3 : 5 an R_f of about 0.92 was observed. Consistent with the formation of mal-sac-spacer-glut was the reaction of the product with iodine vapor, chlorox spray, which is indicative of amide bonds, and a reagent reactive with maleimides, Keller, O. and Rudinger, J., *Helv. Chim. Acta*, 58:531 (1975).

Lastly, maleimido-6-aminocaproyl-spacer-glutaryl ester of 1-hydroxy-2-nitrobenzene sulfonic acid was formed by dissolving 0.2 grams of mal-sac-spacer-glut-OH in 4.0 ml of dimethylformamide, followed by adding 0.534 grams of 1-hydroxy-2-nitrobenzene sulfonic acid and 0.305 grams of dicyclohexylcarbodiimide. The mixture was allowed to react overnight at room temperature, and the active ester product chromatographed over a LH-20 Sephadex column as described above. The identification of fractions containing mal-sac-spacer-glut-HNSA was achieved by spotting a drop of a fraction on to a porcelain plate, followed by the addition of a drop of 5 N NaOH. The latter causes the hydrolysis of the ester, thereby producing a bright yellow color indicative of the HNSA anion. Fractions so identified as to contain mal-sac-spacer-glut-HNSA had the dimethylformamide removed *in vacuo*, and purified on a silica gel Chromatotron 2 mm thick thin-layer-chromatographic plate, using a solvent system consisting of chloroform : methanol : acetic acid, 70 : 30 : 5. The ester containing fractions identified as described previously were pooled and concentrated by rotary evaporation. About 0.113 grams of a pale yellow solid product was obtained which contained 94.1% ester. The product was shown to have a R_f of about 0.25 in chloroform : acetic acid : methanol, 90 : 10 : 10, and an R_f value of about 0.75 in chloroform : methanol : acetic acid, 70 : 30 : 5. Spectral analysis revealed strong absorbance in the UV. Moreover, the product reacted positively with iodine, chlorox spray, and the maleimide reactive reagent. These results indicate that the product is the desired heterobifunctional maleimide active ester crosslinker, maleimido-6-aminocaproyl-spacer-glutaryl ester of 1-hydroxy-2-nitrobenzene sulfonic acid, which has the structure:



Synthesis of Oligonucleotide Antibody Conjugates Through Thioether Bonding

In general, the procedure for forming the oligonucleotide antibody conjugate consists of reacting antibody, either polyclonal or monoclonal, having a free amino group with a maleimide-active ester, produced as described above, in a suitably buffered solution. Preferably, the maleimide-active ester is present in about a two-fold molar excess over antibody, and the pH of the solution is slightly alkaline to maintain the antibody's amino group in an unprotonated state. The reaction of antibody with the thioether crosslinker can be followed by monitoring the absorbance of the solution at a wavelength of about 406 nm. Aldwin & Nitecki, *supra*. An increase in absorbance at this wavelength is the result of the dianion leaving group, HNSA, and the reaction of antibody amines to form stable amide bonds. Because hydrolysis of the crosslinker's active ester is slow relative to aminolysis, most of the leaving groups absorbance is due to amide bond formation. The reaction of antibody with the crosslinker is for a time sufficient to introduce about 0.5-3 crosslinker molecules per antibody molecule. Next, the derivatized antibody is separated from the crosslinker, using any number of standard biochemical separation techniques. Preferably the separation procedure will be accomplished using a gel filtration column, and more preferably Sephadex G-25 (Pharmacia Corp.) will be employed. The column is pre-equilibrated with a chromatographically compatible aqueous buffered solution. The isolated derivatized antibody can then be reacted with the oligonucleotide having a sulfhydryl group as described below.

Oligonucleotide having a free sulfhydryl group can be directly reacted with the derivatized antibody in an aqueous buffered solution compatible with the reaction. The oligonucleotide and antibody concentrations, and the duration of the reaction may vary depending on the number of oligonucleotide molecules sought to be bound to antibody. The reaction is preferably run at 4°C overnight.

Synthesis of the Preferred Oligonucleotide-Antibody Conjugates

More specifically, the synthesis of the more preferred oligonucleotide-antibody conjugate was carried out as follows.

The monoclonal antibody 454A12 was reacted with the heterobifunctional crosslinker, mal-sac-spacer-glut-HNSA as follows. 10 mg/ml of 454A12 was reacted with a two-fold molar excess of the thioether crosslinker in 0.1 M sodium phosphate, pH 8, for about 25 minutes at room temperature. The progress of the reaction was followed by measuring the absorbance as described in Example I at 406 nm. At the end of 25 minutes the absorbance had increased to 0.57, and the derivatized antibody was separated from the reaction mixture by gel filtration using a Sephadex G-25 column

(2.5 x 17 cm) in 40 mM sodium phosphate buffer, pH 6, containing 200 mM NaCl. This material was reacted, as described below, with the oligonucleotide having a reactive sulfhydryl group.

5 The oligonucleotide with reactive sulfhydryl group was combined with derivatized antibody in a 1 : 2 molar ratio (antibody : oligonucleotide with sulfhydryl group). The solution was concentrated using an Amicon stirred ultra-filtration device. The buffer employed was 40 mM sodium phosphate, pH 7.6, containing 200 mM NaCl. The reaction was allowed to proceed overnight at 4°C, and the sample was then chromatographed over GF 250 gel filtration column (PBS pH 7.6). The fractions
10 collected were run on 6.5% SDS-PAGE and conjugates were observed having molecular weight greater than the unconjugated antibody, 150,000 kD, but less than 210,000 kD.

Alternatively, to maximize the conjugation, the oligonucleotide with reactive sulfhydryl group is combined with derivatized antibody in a 1:25 molar ratio (antibody:
15 oligonucleotides with sulfhydryl group). The oligonucleotide and antibody concentrations may vary depending on the number of oligonucleotides bound to the antibody. Additionally, after the reaction has proceeded overnight at 4°C, the sample can be chromatographed over a Sepharose S-300 column (2.2 x 80 cm) in 40 mM sodium phosphate, pH 6.5, containing 200 mM NaCl. This step removed any
20 unreacted oligonucleotides with the sulfhydryl group.

Fractions containing either free antibody, if any, or oligonucleotide antibody conjugate can be identified using a suitable analytical technique, such as sodium dodecyl sulfate polyacrylamide gel electrophoresis. The oligonucleotide antibody conjugates so isolated can, if desired, be concentrated by any suitable technique known
25 in the art followed by sterilization. The latter is readily achieved by passing the oligonucleotide antibody conjugate through an 0.2 micron filter.

The above conjugates would be as efficacious as conjugates formed by disulfide linkages, and the methods of use of both types of conjugates would be similar.

Alternative Conjugates

30 The above specific description of an exemplary conjugate and testing against HIV was presented to illustrate the invention. It should not be construed as limiting the invention. For example, it will be apparent to those skilled in the art that any antisense oligonucleotide that is complementary to the RNA of the retrovirus genomic HIV may be used in the conjugation described above. It has been demonstrated that
35 oligodeoxynucleotides complementary to certain highly conserved regions of the HIV genome inhibit virus replication or gene expression in cultured HIV-transformed human

lymphocytes. U. S. Patent No. 4,806,463, Goodchild et al., February 21, 1989, "Inhibition of HTLV-III by Exogenous Oligonucleotides." That is, oligodeoxynucleotides complementary to (1) sequences 5' to the primary tRNA^{lys} binding site; (2) the primer binding site; (3) sequences of a mRNA donor splice site; or (4) sequences of a mRNA acceptor splice site have been shown to cause inhibition. Id. Examples of the locations on the HIV genome which can serve as the templates for the antisense oligonucleotides are: the tRNA^{lys} primer binding site; regions of the HIV genome vicinal in the 5' direction to the tRNA^{lys} primer binding site; the tRNA^{lys} primer binding site and regions of the HIV genome vicinal in the 5' direction to the tRNA^{lys} primer binding site; the mRNA donor splice sites; the mRNA acceptor splice sites; the initiator codon for the gag gene; the initiator codon for the env gene; the initiator codon for the tat gene; the initiator codon for the sor gene; the initiator codon for the 3' orf gene; the cap nucleotide of the HIV genome; the art gene or portions thereof; the region of the HIV genome encoding a frameshift; the poly (A) addition site; and equivalents thereof. The specific antisense oligonucleotide sequences which can be used are: CTGCTAGAGATddT; TGCTAGAGATTTTCCACAC; TTCAAGTCCCTGTTCTGGGCGCCAAA; GCGTACTCACCAGTCGCCGC; CTGCTAGAGATTAA; ACACCCAATTCTGAAAATGG; CTGGTCTAACCAGAGAGACC; GCAAGCTTTATTGAGGCTTA; and equivalents thereof. Alternatively, the oligonucleotide can target HIV nucleotide sequences which code for protease necessary for proper viral assembly. Oligodeoxynucleotides blocked at the 3' end by ddT, the isourea group, or other chain terminators may prove to be more effective inhibitors. In general, any highly conserved region of the HIV genome which encodes information necessary for viral replication or gene expression (e.g., protein synthesis) is a potential target for complementary oligodeoxynucleotides. Id. Further, the oligonucleotide can be complementary to the viral mRNA, one strand of an integrated or unintegrated proviral DNA, a DNA-RNA, or RNA-RNA duplexes.

Similarly, the antisense oligonucleotide conjugate can be used to inhibit replication or expression of other viruses, for example, herpes viruses in the treatment of herpes. Additionally, in the case of DNA viruses, the oligonucleotides can be complementary to the genomic DNA.

The foregoing general approach of forming conjugates consisting of ligand binding molecule and oligonucleotides may be used to treat diseases other than viral infections, and preferably is applied to the treatment of sepsis, as described below.

TNF Induced Diseases/Symptoms

Examples of antisense oligonucleotides that can be used for preventing or suppressing TNF induced diseases, for example sepsis, are those complementary to TNF DNA or TNF RNA. For example, oligonucleotides complementary to the following can be used: sequences around the 5' end of the TNF messenger RNA; sequences at the beginning of and within the mRNA region coding for the transmembrane domain of the TNF protein; sequence within the coding region of the 17kD molecule. Examples of the specific oligonucleotide sequences complementary to the above mRNA regions are:

10 5'TCTCCCTCTTAGCTGGTCCTCTGC3';
 5'CATGCTTTTCAGTGCTCATGGTGTCTTTC3';
 5'GATCAGGAAGGAGAAGAGGCTGAGGAACAA3';
 5'CTCAGCTTGAGGGTTTGC3'; and 5'TTCGTCCTCCTCACAGGGC3'.

Cancer

15 A third embodiment of the invention is to apply ligand binding molecule-oligonucleotide conjugates to the treatment of cancer, preferable for the treatment of leukemia.

In the treatment of chronic myeloid leukemia (CML), oligodeoxynucleotide that can be used are complementary to the unique fusion sequence of BCR-ABL mRNAs. For example, GCTGAAGGGCTT[^]TTGAACTCTGCTTA, complementary to the BCR exon 3/ABL exon II junction sequence; and GCTGAAGGGCTT[^]CTTCCTTATTGATG complementary to BCR exon 2-ABL exon II fusion sequence. As for the treatment of acute lymphocytic leukemia (ALL), the oligodeoxynucleotide that can be used is GCTGAAGGGCTT[^]CTGCGTCTCCAT, which is complementary to the BCR/ABL junction of the BCR-ABL mRNA.

As applied to the treatment of CML or ALL, the ligand binding molecules can be monoclonal antibodies against leukemia-associated antigens. Examples of these are: anti-CALLA (common acute lymphoblastic leukemia-associated antigen), J5, BA-3, RFB-1, BA-2, SJ-9A4 Du-ALL-1, anti-3-3, anti-3-40, SN1 and CALL2, described in Foon, K.A., et al., 1986, Blood, 68(1):1-31, "Review: Immunologic Classification of Leukemia and Lymphoma". The ligand binding molecules can also be antibodies that identify myeloid cell surface antigens, or antibodies that are reactive with B or T lymphocytes, respectively. Examples of such antibodies are those which identify human myeloid cell surface antigens or those which are reactive with human B or T lymphocytes as described in Foon, K.A., Id. Additional examples are antibodies B43, CD22 and CD19 which are reactive with B lymphocytes can also be used.

Miscellaneous

It will be apparent to those skilled in the art that the oligonucleotide used in the conjugate for the treatment of the above diseases, and other applications, can be either oligodeoxynucleotide or oligoribonucleotide. Among other factors, the choice will be dependent on the ease of synthesis, the efficacy, and the relative stability and special advantages of the oligonucleotides in a particular system. Further, the oligonucleotides can be complementary to either DNA or RNA. It can also bind to either or both single stranded or double stranded nucleic acid. The DNA or RNA can be indigenous (cellular) to the cell in question or they can be foreign nucleic acids found in the host cells. The DNA can be cellular or foreign infectious DNA, e.g., those of virus, bacteria, yeast, fungi and other parasites. The RNA can be genomic RNA or messenger RNA, for example, retroviral genomic RNA, foreign or cellular mRNA. Where the oligonucleotide is complementary to and bound to the genomic DNA or RNA, it inhibits or prevents the nucleic acid from being replicated. By interfering with or inhibiting the replication of the nucleic acid, the oligonucleotide interferes with or inhibits downstream expression of the DNA or RNA in protein synthesis. Where the oligonucleotide is complementary to the messenger RNA it interferes with or inhibits the mRNA from being expressed in protein synthesis.

Further, the ligand binding molecule in the conjugate can be varied. Any ligand binding molecule which facilitates contact of the oligonucleotide with the target cell, or entry of the oligonucleotide into the cells that can be conjugated to the oligonucleotide may be used. Examples of the ligand binding molecule are antibody or growth factor, preferably, interleukin, interleukin antibody and any cell surface recognizing ligand binding molecule and fragments thereof which retain the ability to recognize cell surface molecule. In the treatment of AIDS, the ligand binding molecule can be an antibody against a HIV antigen. In the case of interleukin, the conjugation procedure is essentially the same as that for the conjugation of the oligonucleotide with transferrin receptor antibody.

The conjugates can be used in treating a variety of diseases. Preferably, the conjugates are used in the treatment of AIDS, prevention and treatment of sepsis, and treatment and suppression of tumors, as described in detail below.

Methods for Using the Conjugates in Treatment of Human Viral Infections

As another feature of this invention, there is also disclosed a method of administering the conjugates to a human to treat AIDS, to inhibit the replication of the AIDS virus in infected human cells and/or to prevent AIDS from developing in humans infected with the AIDS virus.

The strategy used in treating a particular individual depends on the status of the individual and the objective of the treatment. For example, an individual who has been found to be carrying the HIV virus but shows no symptoms of AIDS might be treated differently, in terms of both the type of conjugates administered and the dose given, than an individual who has AIDS. In addition, treatment might well differ if its objective is to protect uninfected cells or to have an effect on cells which are already infected.

For example, an individual known to be harboring the virus but yet manifesting no sign of AIDS could be given a long-term or lifetime maintenance dose of the conjugates whose inhibitory effects stop reverse transcription, e.g., by using conjugates with oligonucleotides complementary to the primer binding site and/or sequences close to the primer binding site in the 5' direction. In this instance, to avoid immunologic rejection the ligand binding molecule is preferably human transferrin receptor antibody. In this way, the first step in viral life or replication is inhibited because viral DNA cannot be made and the virus is unable to proliferate. However, in an AIDS patient, cells are already infected and treatment must inhibit expression of viral genes already present in the infected cells. In this case, conjugates comprising oligonucleotides complementary to, for example, initiator codons for genes encoding viral proteins, are required to prevent viral construction. In an AIDS patient, uninfected cells can also be protected by administration of conjugates comprising oligonucleotides capable of blocking reverse transcription. Further, at the early stage of HIV infection, the patient can be administered with conjugates comprising oligonucleotide complementary to the HIV nucleotide sequence which encodes the protease necessary for the viral assembly. By inhibiting the production of protease, the unassembled viral particles will still cause antigenic marker to be expressed on the host cell, but will not be infectious to other cells. Cells expressing such viral antigens then can be immunologically eliminated, either through the patient's own immune response or with other medical treatments. Clearly, such a treatment is viable only where the infected cells are relatively few, such that elimination of these cells will not adversely affect the patients.

Conjugates whose presence in cells can stop reverse transcription and conjugates whose presence in cells can inhibit protein synthesis can be administered by a number of routes, however, intravenous injection is preferred whereby the desirable blood level may be maintained by a continuous infusion or intermittent infusions. The dosage varies with such factors as the size and age of the patient, stage of the disease, the concurrent treatments being given and the type of conjugates used, etc.

For example, the desired dose may be presented as two, three, four or more subdoses administered as infusions at appropriate intervals throughout the day.

Administration is by any suitable route including oral, rectal, nasal, topical (including buccal and sublingual), vaginal and parenteral (including subcutaneous, intramuscular, intravenous and intradermal). It will be appreciated that the preferred route may vary based on the factors discussed in the previous paragraph.

The administered ingredients may be used in therapy in conjunction with other medicaments such as suramin, ribavirin, antimoniotungstate (HPA-23), interferon, e.g., alpha-interferon, interleukin II, AZT, CD4, DDC (dideoxycytidine), DDA (dideoxyadenosine), DDI (dideoxyinosine), lymphokines, casteniosperamine, met-enkephalin, peptide T, dextran sulfate, Imreg-1 and Ampligen, and phosphonoformate (Foscarnet). Further, the conjugates described herein may be used in combination with bone marrow or lymphocyte transplants or other medications such as levamisol or thymosin which would increase lymphocyte numbers and/or function as is appropriate. Where one of the ingredients has side effects, it is given to patients on alternate weeks. For example, AZT causes toxic side effects, such as myelosuppression and severe anemia. DDC is also toxic since it causes serious peripheral neuropathy. Since AZT and DDC exhibit different toxicities, they are given to patients on alternate weeks.

While the administered ingredients may be administered alone, they may be presented as part of a pharmaceutical formulation. The formulations of the present invention comprise at least one conjugate, together with one or more acceptable carriers thereof and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient.

The following is an example of the procedure: the patient is administered intravenous infusion of the above conjugate in a physiologically acceptable carrier at a starting dose of 2.0-20 mg/m² daily for five days. By inhibiting viral expression, host cell decimation by the virus is decreased. The cells, still containing the virus, are treated with chemotherapy administered in conjunction with the conjugate treatment. At the end of the five-day period, the patient is evaluated. The evaluation includes physical examination and extensive laboratory testing. The tests include evaluation for toxicity and specific tests directed to the determination of the T-cell counts. Subjective improvements of the patient are also monitored, e.g., improvement in appetite or strength. If the patient's condition is stable, he is re-treated at the same dosage daily and evaluated weekly. Provided the patient's condition is stable, the treatment may be continued. To avoid an unwanted immune response to the antibody part of the conjugation for long treatment periods, the preferred conjugate consist of human or

humanized antibody coupled to the appropriate oligonucleotide(s). At the end of each period, the patient is again evaluated. Comparison of the pre-treatment and post-treatment T-cell counts indicates the efficacy of the combined treatments by showing whether the viral expression has been inhibited and whether the viral population has decreased. According to the efficacy of the combined treatments, and the patient's condition, the peptide or peptide-ligand binding molecule dosage, the chemotherapy may be increased or maintained constant for the duration of treatment. The patient's condition and the status of the viral expression is monitored periodically through physical exam, and laboratory tests. The starting dose of conjugate and chemotherapy is reduced for a patient who exhibits adverse reaction.

The formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question.

The above is by way of example, and does not preclude methods of treatment that are known by those skilled in the art. It also does not preclude use of the conjugates of treatment of other viral infection, with proper modification according to the specific virus involved.

Methods of Using the Conjugates in Treatment of TNF Induced Diseases/Symptoms

As another feature of this invention, there is also disclosed a method of administering the conjugate to a human to inhibit TNF induced diseases/symptoms, for example, to prevent sepsis, or to inhibit and reduce sepsis after its onset.

Again, the strategy used in treating a particular individual depends on the status of the sepsis and the objective of the treatment. For example, treatments for prevention of sepsis differs from that of containment or reduction after its onset.

For example, an individual without sepsis could be given a maintenance dose of the conjugates to inhibit/stop transcription of the TNF genes. Conjugates comprising oligonucleotides complementary to, for example, initiator codons for the gene encoding TNF can prevent TNF production. Where there is already an onset of sepsis, the conjugates may comprise oligonucleotides complementary to the mRNA coding for TNF. By binding to the mRNA, the conjugates prevent translation of mRNA, therefore, production of TNF is inhibited or stopped. The oligonucleotides that can be utilized in the conjugate are sequences complementary to the 5' end of the TNF messenger RNA; sequences at the beginning of and within the mRNA region coding for transmembrane domain of the TNF protein; and sequences within the coding region of the 17 kD molecule. (Figure 10 shows the locations of these sequences on the HuTNF cDNA.)

The above conjugates can be administered by any number of routes, but preferably by intravenous injection whereby the desirable blood level may be maintained by a continuous infusion or intermittent infusion. The dosage varies with such factors as the size and age of the patient, stage of the disease, the concurrent
5 treatments being given, and the type of conjugates used, etc.

For example, the desired dose may be presented as two, three, four or more subdoses may be infused at appropriate intervals throughout the day. Administration is by any suitable route including oral, rectal, nasal, topical (including buccal sublingual), vaginal and parenteral (including subcutaneous, intramuscular, intravenous and
10 intradermal). It will be appreciated that the preferred route may vary based on the factors discussed in the previous paragraph.

An example of prophylactic use of the conjugate is as follows: 3-5 hours before surgery the patient is administered intravenous infusion of the conjugates in a physiologically acceptable media at a starting dose of 2.0-20 mg/m². The intravenous
15 infusion continues throughout the surgery and 1-10 days post surgery. During this period, the patient's reaction to the conjugate is monitored by means of physical examination, extensive laboratory testing and observation of the patient's subjective reactions. The tests include evaluation for the toxicity, and tests for the level of TNF produced by the patient. According to the efficacy of the treatment, and the patient's
20 condition, the conjugate dosage may be increased, or maintained constant, or decreased for the duration of the treatment.

While the administered ingredients may be administered alone, they may be presented as part of a pharmaceutical formulation. The formulations of the present invention comprise at least one administered conjugate together with one or more
25 acceptable carriers thereof and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient.

Method of Using the Conjugates of Treatment of Human Leukemia

The conjugates can be used for inhibition or suppression of other endogenous
30 gene expression or protein synthesis. For example, the conjugates may be used to suppress expression of oncogenes.

As another feature of this invention, here is also disclosed a method of administering the conjugate to a human to suppress leukemia cause by mutations in the Ph' chromosome.

35 The method is similar to those described in Examples I and II. The modifications being clinical tests for improvement include white blood cells counts.

All the above is by way of example, and does not preclude other conjugates, methods of making the conjugates and methods of treatment using the conjugates that are known by those skilled in the art, or that could be arrived at by those skilled in the art using the guidelines set forth in this specification.

WE CLAIM:

1. A conjugate for treating or preventing disease comprising a cell surface ligand binding molecule conjugated to at least one antisense oligonucleotide, said antisense oligonucleotide being complementary to and capable of hybridizing with a segment of a nucleic acid, said hybridization interfering with or inhibiting the expression or replication of said nucleic acid.
2. A conjugate according to claim 1, wherein the segment of the nucleic acid which is capable of hybridizing to said oligonucleotide is selected from the group consisting of a single stranded DNA, RNA, or a strand belonging to a duplex of DNA-DNA, RNA-RNA, or DNA-RNA.
3. A conjugate according to claim 2, wherein the antisense oligonucleotide(s) comprise oligodeoxynucleotides or oligoribonucleotides.
4. A conjugate according to claim 2, wherein the antisense oligonucleotide(s) further comprise modified oligodeoxynucleotides or oligoribonucleotides.
5. A conjugate according to claim 4, wherein said modified oligonucleotide(s) are selected from the group consisting of: methylphosphonate oligodeoxynucleotide, phosphorothioate oligodeoxynucleotide, 2'-OMe RNA oligonucleotide, α -oligodeoxynucleotide, α -oligodeoxynucleotide covalently linked to intercalating agent(s), oligodeoxynucleotide covalently linked to intercalating agent(s), oligoribonucleotide modified with carbamate, or oligodeoxynucleotide modified with polylysine or iron EDTA or its analogues.
6. A conjugate according to claim 2, wherein the nucleic acid is selected from the group consisting of HIV viral genomic RNA, unintegrated and integrated proviral DNA, viral mRNA, and viral RNA-DNA duplex.
7. A conjugate according to claim 6, wherein said cell surface ligand binding molecule is selected from the group consisting of: growth factors, antibody to growth factors, antibody to growth factor cell surface receptors, and antibody to a complex comprising growth factor and growth factor receptor.

8. A conjugate according to claim 7, wherein said ligand binding molecule is selected from the group consisting of: transferrin, transferrin receptor antibody, interleukin, interleukin antibody, interleukin receptor antibody, and antibody against HIV antigen.

9. A conjugate according to claim 8, wherein said cell surface binding molecule is a transferrin receptor antibody, said transferrin receptor antibody being covalently bound to the antisense oligonucleotide.

10. A conjugate according to claim 8, wherein said antisense oligonucleotide comprises a nucleic acid which is complementary to a region of the HIV genome selected from the group consisting of:

- a) the tRNA^{lys} primer binding site;
- b) regions of the HIV genome vicinal in the 5' direction to the tRNA^{lys} primer binding site;
- c) the tRNA^{lys} primer binding site and regions of the HIV genome vicinal in the 5' direction to the tRNA^{lys} primer binding site;
- d) the mRNA donor splice sites;
- e) the mRNA acceptor splice sites;
- f) the initiator codon for the gag gene;
- g) the initiator codon for the env gene;
- h) the initiator codon for the tat gene;
- i) the initiator codon for the sor gene;
- j) the initiator codon for the 3' orf gene;
- k) the cap nucleotide of the HIV genome;
- l) the art gene or portions thereof;
- m) the region of the HIV genome encoding a frameshift;
- n) the poly (A) region; and
- o) equivalents thereof.

11. A conjugate according to claim 10, wherein said antisense oligonucleotide comprises an oligodeoxynucleotide selected from the group consisting of:

- a) CTGCTAGAGATddT;
- b) CTGCTAGAGATTTTCCACAC;
- c) TTCAAGTCCCTGTTCTGGGCGCCAAA;

- d) GCGTACTCACCAGTCGCCGC;
- e) CTGCTAGAGATTAA;
- f) ACACCCAATTCTGAAAATGG;
- g) CTGGTCTAACCAGAGAGACC;
- h) GCAAGCTTTATTGAGGCTTA; and
- g) equivalents thereof.

12. A method of inhibiting HIV replication, HIV gene expression or both in an individual, comprising administering to said individual an effective amount of a conjugate comprising a cell surface ligand binding molecule conjugated to at least one antisense oligonucleotide; said antisense oligonucleotide being complementary to and capable of hybridizing with a segment of a HIV nucleic acid selected from the group consisting of viral genomic RNA, unintegrated and integrated proviral DNA, viral mRNA, and viral RNA-DNA duplex.

13. A method according to claim 12, wherein said cell surface ligand binding molecule is selected from the group consisting of growth factor, antibody to growth factor, antibody to growth factor receptor, and antibody capable of recognizing a complex comprising growth factor and growth factor receptor.

14. A method according to claim 13, wherein said cell surface ligand binding molecule is selected from the group consisting of: transferrin, transferrin receptor antibody, interleukin, interleukin antibody, interleukin receptor antibody, and antibody to HIV antigen.

15. A method according to claim 14, wherein said antisense oligonucleotide comprises a nucleic acid which is complementary to a region of the HIV genome selected from the group consisting of:

- a) the tRNA^{lys} primer binding site;
- b) regions of the HIV genome vicinal in the 5' direction to the tRNA^{lys} primer binding site;
- c) the tRNA^{lys} primer binding site and regions of the HIV genome vicinal in the 5' direction to the tRNA^{lys} primer binding site;
- d) the mRNA donor splice sites;
- e) the mRNA acceptor splice sites;
- f) the initiator codon for the gag gene;

- g) the initiator codon for the env gene;
- h) the initiator codon for the tat gene;
- i) the initiator codon for the sor gene;
- j) the initiator codon for the 3' orf gene;
- k) the cap nucleotide of the HIV genome;
- l) the art gene or portions thereof;
- m) the region of the HIV genome encoding a frameshift;
- n) the poly (A) region; and
- o) equivalents thereof.

16. A method according to claim 15, wherein the antisense oligonucleotide comprises oligodeoxynucleotides selected from the group consisting of:

- a) CTGCTAGAGATddT;
- b) CTGCTAGAGATTTCCACAC;
- c) TTCAAGTCCCTGTTCTGGGCGCCAAA;
- d) GCGTACTCACCAGTCGCCGC;
- e) CTGCTAGAGATTAA;
- f) ACACCCAATTCTGAAAATGG;
- g) CTGGTCTAACCAGAGAGACC;
- h) GCAAGCTTTATTGAGGCTTA; and
- g) equivalents thereof.

17. A conjugate according to claim 2, wherein the antisense oligonucleotide is complementary to segments of TNF DNA or TNF RNA.

18. A conjugate according to claim 17, wherein said antisense oligonucleotide is selected from the group consisting of:

- a) 5'TCTCCCTCTTAGCTGGTCCTCTGC3';
- b) 5'CATGCTTTCAGTGCTCATGGTGTCTTTC3';
- c) 5'GATCAGGAAGGAGAAGAGGCTGAGGAACAA3';
- d) 5'CTCAGCTTGAGGGTTTGC3'; and
- e) 5'TTCGTCCTCCTCACAGGGC3'.

19. A conjugate according to claim 18, wherein said cell surface ligand binding molecule is selected from the group consisting of: growth factor, antibody to growth factor, antibody to cell surface receptor and antibody capable of recognizing complex comprising growth factor and growth factor receptor.

20. A method of preventing or suppressing TNF induced diseases or symptoms in an animal, comprising administering to said animal an effective amount of a conjugate comprising a cell surface ligand binding molecule conjugated to at least one antisense oligonucleotide; said antisense oligonucleotide being complementary to and capable of hybridizing with a segment of a TNF RNA or TNF DNA.

21. A conjugate according to claim 2, wherein the antisense oligonucleotide is complementary to BCR-ABL messenger RNA.

22. A conjugate according to claim 21, wherein said antisense oligonucleotide is selected from the group consisting of:

- a) GCTGAAGGGCTT[^]TTGAACTCTGCTTA;
- b) GCTGAAGGGCTT[^]CTTCCTTATTGATG;
- c) GCTGAAGGGCTT[^]CTGCGTCTCCAT; and
- d) equivalents thereof.

23. A conjugate according to claim 22, wherein said cell surface ligand binding molecule is selected from the group consisting of growth factor, antibody to growth factor, antibody to cell surface receptor, antibody capable of recognizing complex comprising growth factor and growth factor receptor, antibody to leukemia associated antigen.

24. A method for treating leukemia comprising administering an effective amount of a conjugate comprising cell surface ligand binding molecule conjugated to at least one antisense oligonucleotide, said antisense oligonucleotide being complementary to and capable of hybridizing with a segment of BCR-ABL mRNA.

25. A method according to claim 24, wherein said antisense oligonucleotides is selected from the group consisting of:

- a) GCTGAAGGGCTT[^]TTGAACTCTGCTTA;
- b) GCTGAAGGGCTT[^]CTTCCTTATTGATG;
- c) GCTGAAGGGCTT[^]CTGCGTCTCCAT; and
- d) equivalents thereof.

26. A method of making a conjugate comprising at least one oligonucleotide linked to a ligand binding molecule by a disulfide linkage, comprising the steps of:

- a) adding a sulfhydryl group to the ligand binding molecule to form a sulfhydryl-ligand binding molecule complex;
- b) adding a sulfhydryl group to the oligonucleotide to form a sulfhydryl-oligonucleotide complex;
- c) reacting said sulfhydryl-ligand binding molecule complex with said sulfhydryl-oligonucleotide complex to form said conjugates; and
- d) isolating said conjugate.

27. A method of making a conjugate comprising at least one oligonucleotide linked to a ligand binding molecule by a thioether linkage, comprising the steps of:

- a) forming a ligand binding molecule complex comprising reacting said ligand molecule with a heterobifunctional crosslinker comprising an activated carboxylate group, a spacer molecule containing either groups, and a maleimide group, wherein an amino group on said ligand binding molecule reacts with said activated carboxylate group;
- b) isolating said ligand binding molecule crosslinker complex;
- c) adding a sulfhydryl group to an oligonucleotide to form a sulfhydryl-oligonucleotide complex;
- d) reacting said sulfhydryl oligonucleotide complex with said ligand binding molecule crosslinker complex to form said conjugates; and
- e) isolating said conjugate.

1 / 8

FIG. 1

BB01	20-MER	
CTGGTCTAACCAGAGAGACC		
MW AMMONIUM SALT		6417.65
MOLAR EXTINCTION AT 260nm		195800
MICROGRAMS PER OD260nm		32.78
PICOMOLES PER OD260nm		5107.25
BASE COMPOSITION: ACGT		6653
Td (blot) 0.1M Na+		62
Tm @ 0.1M Na+, .000001M Probe		58

BB02	20-MER	
GCAAGCTTTATTGAGGCTTA		
MW AMMONIUM SALT		6453.67
MOLAR EXTINCTION AT 260nm		193700
MICROGRAMS PER OD260nm		33.32
PICOMOLES PER OD260nm		5162.62
BASE COMPOSITION: ACGT		5357
Td (blot) 0.1M Na+		56
Tm @ 0.1M Na+, .000001M Probe		60

BB03	20-MER	
CAGTCAGTCAGTCAGTCAGT		
MW AMMONIUM SALT		6423.65
MOLAR EXTINCTION AT 260nm		196900
MICROGRAMS PER OD260nm		32.62
PICOMOLES PER OD260nm		5078.72
BASE COMPOSITION: ACGT		5555
Td (blot) 0.1M Na+		60
Tm @ 0.1M Na+, .000001M Probe		55

2 / 8

FIG. 2

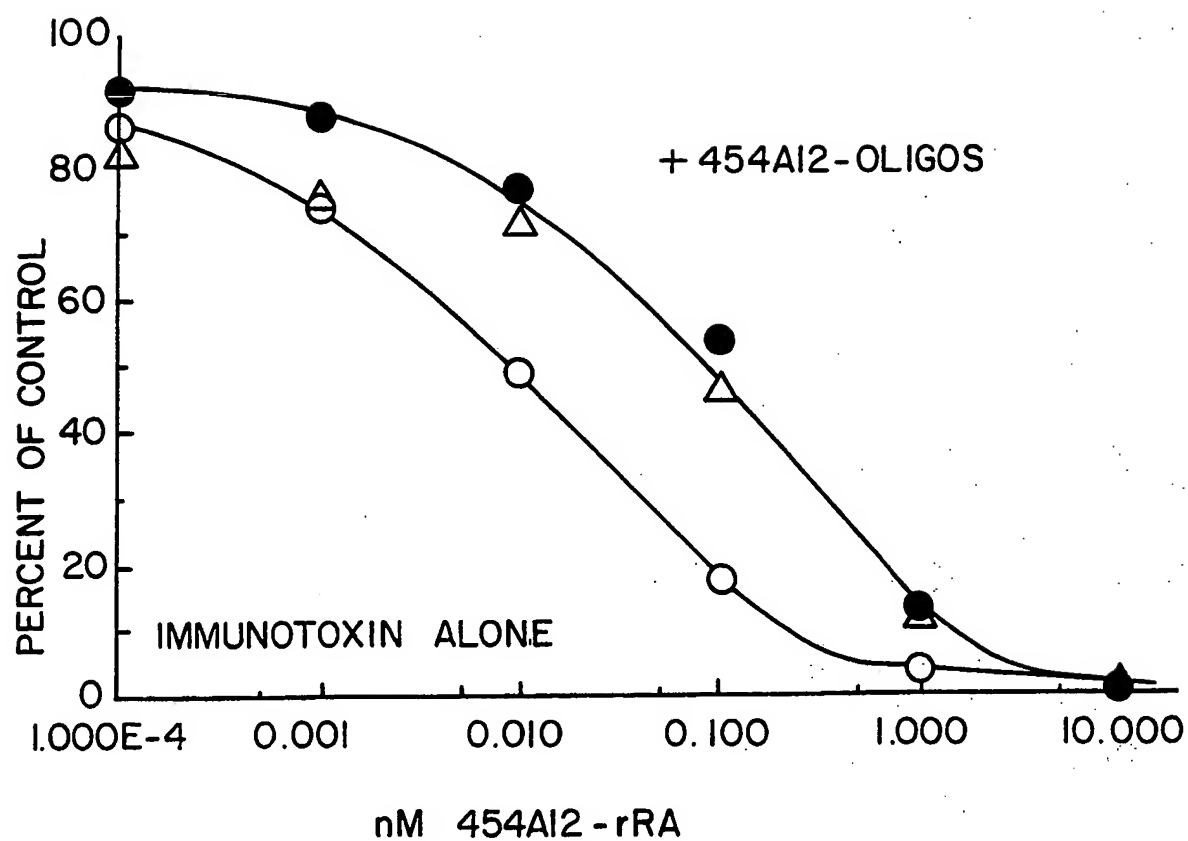
BB04 21-MER
 XCTGGTCTAACCAGAGAGACC
 MW AMMONIUM SALT 6417.65
 MOLAR EXTINCTION AT 260nm 220400
 MICROGRAMS PER OD260nm 29.12
 PICOMOLES PER OD260nm 4537.21
 BASE COMPOSITION: ACGT 6653
 MIXED BASES: YRNMKSWHBVDXZ 0000000000010
 X=ANTITRANSFERRIN RECEPTOR ANTIBODY,
 Td (blot) 0.1M Na+ 63
 Tm @ 0.1M Na+, .000001M Probe 58

BB05 21-MER
 XGCAAGCTTTATTGAGGCTTA
 MW AMMONIUM SALT 6453.67
 MOLAR EXTINCTION AT 260nm 217600
 MICROGRAMS PER OD260nm 29.66
 PICOMOLES PER OD260nm 4595.59
 BASE COMPOSITION: ACGT 5357
 MIXED BASES: YRNMKSWHBVDXZ 0000000000010
 X=ANTITRANSFERRIN RECEPTOR ANTIBODY,
 Td (blot) 0.1M Na+ 57
 Tm @ 0.1M Na+, .000001M Probe 60

BB06 21-MER
 XCAGTCAGTCAGTCAGTCAGT
 MW AMMONIUM SALT 6423.65
 MOLAR EXTINCTION AT 260nm 215000
 MICROGRAMS PER OD260nm 29.88
 PICOMOLES PER OD260nm 4651.16
 BASE COMPOSITION: ACGT 5555
 MIXED BASES: YRNMKSWHBVDXZ 0000000000010
 X=ANTITRANSFERRIN RECEPTOR ANTIBODY,
 Td (blot) 0.1M Na+ 61
 Tm @ 0.1M Na+, .000001M Probe 55

3/ 8

FIG. 3



4 / 8

FIG. 4

Reverse transcriptase levels (cmp/0.01 ml) at 12 days in culture fluids of HIV-infected macrophages cocultivated with:

sample	concentration		
1*	1/100	1/1000	1/10000
2	900	1200	1300
3	1200	3000	7100
4	12,100	35,700	34,000
5	36,600	30,100	15,300
6	5100	6100	3200
7*	1900	1800	2200
8*	600	800	900
9	900	900	1000
10	2700	7100	11240
	2000	6800	38400
medium	6200		
background	1100		

5 / 8

FIG. 5

sample	day 5	Reverse transcriptase cpm day 7	Reverse transcriptase cpm day 12
medium	6700	166000	240000
1	1200	78000	356000
5	1300	85000	138000
6	1400	46000	167000
7	1400	27000	74000
8	800	1300	3500
background	1300		

6 / 8

5 ① GCAGAGGACCAGCTAAGAGGGAGAGAAGCAACTACAGACCCCCCTGAAAACAACCCTC
50

AGACGCCACATCCCCTGACAAGCTGCCAGGCAGGTTCTCTTCTCTCACATACTGACCCAC
100

GGCTCCACCCTCTCTCCCCTGGAAAGGACACC
② 150

-76
met ser thr glu ser met
ATG AGC ACT GAA AGC ATG

-70
ile arg asp val glu leu ala glu glu ala leu pro lys lys thr
ATC CGG GAC GTG GAG CTG GCC GAG GAG GCG CTC CCC AAG AAG ACA
200

-50
gly gly pro gln gly ser arg arg cys leu phe leu ser leu phe
GGG GGG CCC CAG GGC TCC AGG CGG TGC TTG TTC CTC AGC CTC TTC
② 250

-40
ser phe leu ile val ala gly ala thr thr leu phe cys leu leu
TCC TTC CTG ATC GTG GCA GGC GCC ACC ACG CTC TTC TGC CTG CTG
300

-20
his phe gly val ile gly pro gln arg glu glu phe pro arg asp
CAC TTT GGA GTG ATC GGC CCC CAG AGG GAA GAG TTC CCC AGG GAC
350

-10
leu ser leu ile ser pro leu ala gln ala Val Arg Ser Ser Ser
CTC TCT CTA ATC AGC CCT CTG GCC CAG GCA GTC AGA TCA TCT TCT

10
Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Val Ala Asn Pro
CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT GTT GTA GCA AAC CCT
400 ③ 20

30
Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala
CAA GCT GAG GGG CAG CTC CAG TGG CTG AAC CGC CGG GCC AAT GCC
450

40
Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu Val Val
CTC CTG GCC AAT GGC GTG GAG CTG AGA GAT AAC CAG CTG GTG GTG
500

60
Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys
CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC TTC AAG
550

FIG. 6-1

7/ 8

70
 Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 GGC CAA GGC TGC CCC TCC ACC CAT GTG CTC CTC ACC CAC ACC ATC
 600

90
 Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTC AAC CTC CTC TCT
 650

100
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Ile
 GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG
 700

120
 Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln
 GCC AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG
 750

130
 Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp
 CTG GAG AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC
 800

150
 Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile
 TAT CTC GAC TTT GCC GAG TCT GGG CAG GTC TAC TTT GGG ATC ATT

③ 157
 Ala Leu OP
 GCC CTG TGA GGAGGACGAACATCCAACCTTCCCAAACGCCTCCCCTGCCCAATCCC
 850 900

TTTATTACCCCTCCTTCAGACACCCTCAACCTCTTCTGGCTCAAAAAGAGAATTGGGGG
 950

CTTAGGGTCGGAACCCAAGCTTAGAACTTTAAGCAACAAGACCACCACTTCGAAACCTGG
 1000

GATTCAGGAATGTGTGGCCTGCACAGTGAATTGCTGGCAACCACTAAGAATTCAAACCTGG
 1050

GGCCTCCAGAACTCACTGGGGCCTACAGCTTTGATCCCTGACATCTGGAATCTGGAGACC
 1100

AGGGAGCCTTTGGTTCTGGCCAGAATGCTGCAGGACTTGAGAAGACCTCACCTAGAAATT
 1150 1200

GACACAAGTGGACCTTAGGCCTTCCTCTCTCCAGATGTTTCCAGACTTCCTTGAGACACG
 1250

GAGCCCAGCCCTCCCATGGAGCCAGCTCCCTCTATTTATGTTTGAAGTGTGATTATTT
 1300

FIG. 6-2

8 / 8

ATTATTTATTTATTATTTATTTATTTACAGATGAATGTATTTATTTGGGAGACCGGGGTA
1350
TCCTGGGGGACCCAATGTAGGAGCTGCCTTGGCTCAGACATGTTTTCCGTGAAAACGGAG
1400
CTGAACAATAGGCTGTTCCCATGTAGCCCCCTGGCCTCTGTGCCTTCTTTTGATTATGTT
1450 1500
TTTTAAAATATTTATCTGATTAAGTTGTCTAAACAATGCTGATTTGGTGACCAACTGTCA
1550
CTCATTGCTGAGCCTCTGCTCCCCAGGGGAGTTGTGTCTGTAATCGCCCTACTATTCAGT
1600
GGCGAGAAATAAAGTTTGCTT

- 1) Sequences around the 5' end of the TNF messenger RNA.
- 2) Sequences at the beginning of and within the mRNA region coding for transmembrane domain of the TNF protein.
- 3) Sequences within the coding region of the 17 kD molecule.

FIG. 6-3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 90/05272

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC <div style="margin-top: 10px;"> IPC⁵: A 61 K 47/48 </div>																	
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="height: 40px; vertical-align: top; border-right: 1px solid black;"> <div style="font-size: 1.2em;">IPC⁵</div> </td> <td style="vertical-align: top;"> <div style="font-size: 1.2em;">A 61 K</div> </td> </tr> </table> <div style="text-align: center; margin-top: 10px; font-size: 0.8em;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ </div>			Classification System	Classification Symbols	<div style="font-size: 1.2em;">IPC⁵</div>	<div style="font-size: 1.2em;">A 61 K</div>											
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<div style="font-size: 1.2em;">IPC⁵</div>	<div style="font-size: 1.2em;">A 61 K</div>																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ¹⁰</th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;"> WO, A, 88/05077 (BATTELLE MEMORIAL INSTITUTE) 14 July 1988 see page 2, paragraph 3 - page 12, paragraph 3; page 18 - page 21, fourth embodiment; page 24 - page 25, sixth embodiment; page 43, paragraph 5 </td> <td style="vertical-align: top;"> 1-4, 6-9, 26, 27 </td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="text-align: center; vertical-align: top;">--</td> <td style="vertical-align: top;"> 1-11, 17-19, 21-23, 26, 27 </td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;"> EP, A, 0263740 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)) 13 April 1988 see page 2, line 59 - page 5, line 44; page 6. lines 45-65 </td> <td style="vertical-align: top;"> 1-5 </td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;"> Proc. Natl. Acad. Sci. USA, volume 85, August 1988, (Washington, DC, US), J. Goodchild et al.: "Inhibition of human immunodeficiency virus </td> <td style="vertical-align: top;"> 1-4, 6-11, 26 </td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	WO, A, 88/05077 (BATTELLE MEMORIAL INSTITUTE) 14 July 1988 see page 2, paragraph 3 - page 12, paragraph 3; page 18 - page 21, fourth embodiment; page 24 - page 25, sixth embodiment; page 43, paragraph 5	1-4, 6-9, 26, 27	Y	--	1-11, 17-19, 21-23, 26, 27	X	EP, A, 0263740 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)) 13 April 1988 see page 2, line 59 - page 5, line 44; page 6. lines 45-65	1-5	Y	Proc. Natl. Acad. Sci. USA, volume 85, August 1988, (Washington, DC, US), J. Goodchild et al.: "Inhibition of human immunodeficiency virus	1-4, 6-11, 26
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; vertical-align: top;"> Date of the Actual Completion of the International Search <div style="text-align: center; margin-top: 10px; font-size: 1.1em;">28th January 1991</div> </td> <td style="width: 50%; border-bottom: 1px solid black; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: center; margin-top: 10px; font-size: 1.1em;">26 FEB 1991</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; vertical-align: top;"> International Searching Authority <div style="text-align: center; margin-top: 10px; font-weight: bold;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; vertical-align: top;"> Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> MISS TAZELAAR </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; margin-top: 10px; font-size: 1.1em;">28th January 1991</div>	Date of Mailing of this International Search Report <div style="text-align: center; margin-top: 10px; font-size: 1.1em;">26 FEB 1991</div>	International Searching Authority <div style="text-align: center; margin-top: 10px; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> MISS TAZELAAR </div>											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	<p>replication by antisense oligodeoxy- nucleotides", pages 5507-5511 see page 5507, abstract; page 5509, table 1 (cited in the application)</p> <p>--</p>	
Y	<p>Proc. Natl. Acad. Sci. USA, volume 86, June 1989, (Washington, DC, US), M. Matsukura et al.: "Regulation of viral expression of human immuno- deficiency virus in vitro by an anti- sense phosphorothioate oligodeoxy- nucleotide against rev (art/trs) in chronically infected cells", pages 4244-4248 see page 4244, abstract; page 4245, figure 1 (cited in the application)</p> <p>--</p>	1-11,26
Y	<p>Science, volume 228, 12 April 1985, (Washington, DC, US), A.M. Wang et al.: "Molecular cloning of the complementary DNA for human tumor necrosis factor", pages 149-154 see page 150, abstract; page 151, paragraphs 2-6; page 152, figure 4B</p> <p>--</p>	1-4,6-9, 17-19,26
Y	<p>Proc. Natl. Acad. Sci. USA, volume 85, August 1988, (Washington, DC, US), E.S. Kawasaki et al.: "Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia- specific mRNA sequences amplified in vitro", pages 5698-5702 see page 5698, abstract; page 5699, paragraphs 3-5 (cited in the application)</p> <p>--</p>	1-4,21-23,26
Y	<p>Proc. Natl. Acad. Sci. USA, volume 85, October 1988, (Washington, DC, US), P.S. Sarin et al.: "Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleoside methylphos- phonates", pages 7448-7451 see page 7448, abstract; page 7450, table 1 (cited in the application)</p> <p>--</p>	1-11,26
	--	./..

III: DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	File Server STN, File Chemical Abstracts, AN no. CA107(21)194341h, American Chemical Society, L. Aldwin et al.: "A water-soluble, monitorable peptide and protein cross- linking agent", & Anal. Biochem., 164(2), 494-501, 1987 see the abstract --	27
Y	EP, A, 0227351 (SHIONOGI AND CO. LTD) 1 July 1987 see column 2, lines 31-43; column 3, line 32 - column 4, line 20 --	27
P,X	Proc. Natl. Acad. Sci. USA, volume 87, April 1990, (Washington, DC, US), J.-P. Leonetti et al.: "Antibody- targeted liposomes containing oligodeoxyribonucleotides complementary to viral RNA selectively inhibit viral replication", pages 2448-2451 see page 2448, abstract --	1-3
A	US, A, 4545985 (PASTAN et al.) 8 October 1985 see claims 1-8 -----	9

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers **, because they relate to subject matter not required to be searched by this Authority, namely:

** Claim numbers 12-16, 20, 24, 25

See Rule 39.1(iv): methods for treatment of the human or animal body by surgery or therapy, as well diagnostic methods,

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9005272

SA 40481

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 19/02/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8805077	14-07-88	None	
EP-A- 0263740	13-04-88	FR-A,B 2604438 JP-A- 63101395	01-04-88 06-05-88
EP-A- 0227351	01-07-87	JP-A- 62132172 GB-A,B 2184127	15-06-87 17-06-87
US-A- 4545985	08-10-85	None	

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